Effector MiSSP7 of the mutualistic fungus *Laccaria bicolor* stabilizes the *Populus* JAZ6 protein and represses jasmonic acid (JA) responsive genes

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Ectomycorrhizal fungi, such as *Laccaria bicolor*, support forest growth and sustainability by providing growth-limiting nutrients to their plant host through a mutualistic symbiotic relationship with host roots. We have previously shown that the effector protein MiSSP7 (Mycorrhiza-induced Small Secreted Protein 7) encoded by *L. bicolor* is necessary for the establishment of symbiosis with host trees, although the mechanistic reasoning behind this role was unknown. We demonstrate here that MiSSP7 interacts with the host protein PtJAZ6, a negative regulator of jasmonic acid (JA)-induced gene regulation in *Populus*. As with other characterized JASMONATE ZIM-DOMAIN (JAZ) proteins, PtJAZ6 interacts with PCO1 in the presence of the JA mimick coronatine, and PtJAZ6 is degraded in plant tissues after JA treatment. The association between MiSSP7 and PtJAZ6 is able to protect PtJAZ6 from this JA-induced degradation. Furthermore, MiSSP7 is able to block—or mitigate—the impact of JA on *L. bicolor* colonization of host roots. We show that the loss of MiSSP7 production by *L. bicolor* can be complemented by transgenically varying the transcription of *PtJAZ6* or through inhibition of JA-induced gene regulation. We conclude that *L. bicolor*, in contrast to arbuscular mycorrhizal fungi and biotrophic pathogens, promotes mutualism by blocking JA action through the interaction of MiSSP7 with PtJAZ6.

Significance

Plants use the hormone jasmonic acid (JA) to modulate plant: microbe interactions. Disease-causing microbes use proteins to alter host JA signaling to aid their growth in plant tissues. Beneficial symbiotic fungi, which colonize plant tissues and provide essential ecosystem services such as carbon sequestration and plant fertilization, can also alter JA signaling in plant cells to promote colonization. Here, we demonstrate that the MiSSP7 (Mycorrhiza-induced small secreted protein-7) protein of the beneficial fungus *Laccaria bicolor* interacts with host plant JA signaling repressors and, in contrast to biotrophic pathogens, promotes symbiosis by blocking JA action. These results shed new light on how beneficial and pathogenic microbes have evolutionarily diverged in the mechanisms by which they overcome plant defenses.


The authors declare no conflict of interest.

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by the hyphae of biotrophic fungi, is inhibited by increased levels of JA (19). How the ECM fungus is able to aggressively colonize plant tissues and not be repulsed by plant defenses controlled by JA and other plant hormones is not well understood.

Because pathogenic organisms favor the use of secreted “effector” proteins to subvert host immunity, a great deal of research has focused on the role of pathogenic effectors and how they are used to modify the signaling of defense pathways controlled by plant hormones (10–12, 20–24). For example, the pathogenic bacteria *Pseudomonas syringae* and the oomycete *Hyaloperonospora arabidopsidis* secrete a number of effectors that target either JAZ proteins (10, 11, 23) or ethylene signaling (24), whereas *Phytophthora infestans* produces different effectors that interfere with SA-related signaling (21, 22). Like pathogenic organisms, the ECM fungus *Laccaria bicolor* also produces effector proteins called MiSSPs (Mycorrhiza-induced Small Secreted Proteins) (25). The first MiSSP of *L. bicolor* to be characterized, MiSSP7, was found to enter host cells and localize to the nucleus where it altered host cell transcription. Localization of MiSSP7 in host cell nuclei was found to be essential for the formation of the Hartig net, although the mechanistic reasoning behind this effect was unknown (26). Here we demonstrate that MiSSP7 interacts with the transcriptional repressor protein PtJAZ6 in the nuclei of the host plant *Populus trichocarpa,* where it protects PtJAZ6 from JA-induced degradation. Furthermore, MiSSP7 is able to counter the negative impacts of JA on fungal colonization of host tissues by repression of JA-induced gene transcription, likely through its interaction with JAZ proteins. Our results further support the concept that, like pathogenic organisms, mutualistic fungi use effectors to target plant host hormone pathways to foster fungal colonization.

**Results**

As reported previously, *L. bicolor* MiSSP7 is able to enter roots of its host *P. trichocarpa* and alter transcription therein (26). Using a yeast reporter assay (27), we tested if the mature version of MiSSP7 (without a signal peptide) could bind to DNA and induce gene transcription. We found that MiSSP7 alone was not able to induce expression of the reporter genes (Fig. S1), demonstrating that the MiSSP7 protein itself does not directly activate the differential gene transcription previously observed (26). Therefore, we used a yeast two-hybrid assay (YTH) (27) to determine the protein target of MiSSP7 in poplar root cells. In this particular version of YTH, a positive interaction between two proteins per-
transcriptional repressors of the JA pathway, we tested the ability of the presence of a JA-Ile mimic. Because JAZ proteins are trans- PtCOI1 and PtJAZ6 led us to test whether or not coronatine induces JA signaling (Fig. S5 and Table S1). Therefore, PtJAZ6 negatively interferes with coronatine-induced binding of PtCOI1 to PtJAZ6 (Fig. 3A). In both assays only PtCOI1 was found to interact with PtJAZ6 and only in the presence of coronatine (Fig. 2 A–D). Furthermore, the strength of the interaction between PtJAZ6:PtCOI1 was increased in yeast cells grown under higher levels of coronatine as determined by a concomitant increase in the levels of β-galactosidase activity (Fig. 2 E and F). Because coronatine did not affect the growth rate of these cells (Fig. S4 A) nor did it promote the interaction between two proteins from a pathway that is unrelated to JA-Ile/coronatine (i.e., negative control for coronatine promotion of protein:protein interactions) (Fig. S4 B), these results indicate that, like the Arabidopsis model, PtJAZ6 interacts with PtCOI1 but only in the presence of a JA-Ile mimic. Because JAZ proteins are transcriptional repressors of the JA pathway, we tested the ability of PtJAZ6 to repress the expression of JA marker genes (26). Transgenic poplar roots with increased expression of PtJAZ6 resulted in the repression of gene transcription associated with JA signaling (Fig. S5 and Table S1). Therefore, PtJAZ6 negatively regulates part of the JA pathway in poplar roots.

The result that coronatine induces the interaction between PtCOI1 and PtJAZ6 led us to test whether or not coronatine could also increase the strength of the interaction between MiSSP7 and PtJAZ6 as quantified by β-galactosidase activity of the yeast cells (Fig. 3 A). At all concentrations tested, however, coronatine had no visible effect on promoting the interaction between these proteins. Rather, as concentrations increased, coronatine significantly disrupted the interaction between MiSSP7 and PtJAZ6 as demonstrated by a significant decrease in β-galactosidase activity (Fig. 3 A). Thus, the interaction between MiSSP7 and PtJAZ6 is not stimulated by a mimic of JA-Ile. Furthermore, we tested the ability of MiSSP7 to alter coronatine-induced binding between PtCOI1 and PtJAZ6. We found that MiSSP7, which is taken up by yeast cells and localizes to the nucleus in plants (Fig. S4 C), significantly decreased the ability of PtCOI1 to interact with PtJAZ6 in the presence of coronatine (Fig. 3B). Because our findings above demonstrate that MiSSP7 interferes with coronatine-induced binding of PtCOI1 to PtJAZ6, we investigated whether MiSSP7 could inhibit JA-induced degradation of PtJAZ6 in planta. In this experiment we expressed PtJAZ6-GFP and MiSSP7 in Nicotiana benthamiana and treated the leaves with MeJA or buffer. GFP fluorescence in nuclei of epidermal cells was quantified. Treatment with MeJA alone resulted in the almost complete loss of nuclear fluorescence indicating degradation of the protein PtJAZ6-GFP (Fig. 3C), but leaves coexpressing MiSSP7 inhibited the loss of PtJAZ6-GFP fluorescence (Fig. 3C). These results were confirmed by Western blotting (Fig. 3D). Taken together, these data would suggest that MiSSP7 is interfering with the JA-induced degradation of the PtJAZ6-GFP fusion protein in the nucleus thereby resulting in an increased lifetime for PtJAZ6.

Because MiSSP7 interacts with PtJAZ6, a repressor of JA-induced gene expression, and as MiSSP7 increases the lifetime of PtJAZ6 in the nucleus during JA treatment, it would thus follow that MiSSP7 treatment of poplar roots should result in the repression of JA-inducible genes. We tested this hypothesis by analyzing the expression of known JA-inducible genes (29) in the roots of poplar heterologously overexpressing MiSSP7. We found that, of the 23 JA-inducible genes tested, 11 were significantly repressed in at least one of the transgenic lines expressing MiSSP7 and mainly consisted of genes coding for cell wall active enzymes (e.g., extensin, pectinesterase, chitinase) (Fig. 3E and
treated/uncolonized poplar roots (fold-change obtained from RNA-seq data; the other datapoints were obtained by comparing *L. bicolor* transgenic lines expressing MiSSP7 (gray and black bars) and in roots colonized by wild-type *L. bicolor* (stippled bars). Ratio of gene expression in MeJA-treated tissues was reinstated at concentrations of MeJA that would normally be inhibiting this process (i.e., 10^{-12} M) having no significant effect on the establishment of the Hartig net and concentrations higher than 10^{-12} M significantly inhibiting Hartig net formation (Fig. 4A). The inhibition of Hartig net establishment was not a result of reduced fungal growth rate caused by MeJA addition (Fig. S6).

When 15 μM synthetic MiSSP7 was exogenously added to the root system undergoing colonization by *L. bicolor* in the presence of MeJA, however, the development of a Hartig net was reestablished at concentrations of MeJA that would normally inhibit this process (i.e., 10^{-10} M) (Fig. 4A). Because MiSSP7 is able to inhibit JA activity during Hartig net formation, and as lack of MiSSP7 expression during root colonization stops Hartig net formation (26), it is possible that the lack of MiSSP7 expression during the colonization process could be complemented by either increasing the expression of *PtJAZ6* or by inhibiting JA-induced gene transcription. To test this hypothesis, we generated transgenic roots with increased or decreased production of *PtJAZ6* and tested the ability of L. *bicolor* to form a Hartig net in these roots (Fig. 4B and Figs. S6 and S7). We found that in *PtJAZ6* RNAi lines, the development of the Hartig net was significantly impeded in roots being colonized by wild-type *L. bicolor* (Fig. 4B). Conversely, formation of a Hartig net in *L. bicolor Δmissp7* lines was found to be reestablished if *PtJAZ6* was overexpressed in root tissues (>2-fold) (Fig. 4C and Fig. S7). The observed establishment of the Hartig net in 35S::*PtJAZ6* roots colonized *L. bicolor Δmissp7* was abolished by the external application of MeJA, indicating that the phenotype was a result of altered JA signaling rather than an artifact of the transformation process (Fig. 4C). To complement these transgenic assays, we tested whether the previously described inhibitors of JA-induced gene expression, SHAM and aspirin (30), could complement the *L. bicolor Δmissp7* RNAi mutant. Because these inhibitors have not been verified as repressors of the JA pathway in poplar, we analyzed the ability of both compounds to repress JA-induced gene transcription. We found that both inhibitors significantly repressed JA-induced gene expression patterns (Fig. S8). We also found that both SHAM and aspirin application to roots led to the formation of a Hartig net by *L. bicolor Δmissp7* RNAi lines. Therefore, an increase in the expression of *PtJAZ6* or the pharmacological suppression of JA-induced gene expression are each able to replace the role of MiSSP7 during the development of the Hartig net.

**Discussion**

Plants have developed a complex defensive response system to protect themselves against invasion by detrimental organisms, often mediated by plant hormones. In turn, invading organisms have developed various methods to circumvent the plant's defenses or control plant cell function to their benefit. Pathogens, such as *P. syringae* and *H. arabidopsidis*, attempt to manipulate the plant response by producing effectors that target different components of the JA (i.e., JAZ proteins) and ET signaling pathways (i.e., ERF proteins) in such a fashion that colonization is favored (10, 11, 23, 24). Like pathogenic bacteria, mutualistic fungi affect plant hormone signaling cascades to achieve colonization (31–35), although the knowledge of the mechanistic reasoning behind most of these differences is in its infancy. We demonstrate here that MiSSP7, an effector protein produced by

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Table S1). It was not expected that all of the genes tested would be repressed because MiSSP7 is only able to interact with two JAZ proteins of poplar and JAZ proteins have been demonstrated to have discrete functions (8). We further tested the expression of these JAZ marker genes in roots colonized by *L. bicolor* (Fig. 3E).

We found that of the 23 genes tested, 10 were significantly repressed in colonized root tissues. Six of these genes were also down-regulated by MiSSP7 and were found to be homologous to extensin, peroxidase, and pectinesterase genes (Fig. 3E and Table S1). Therefore, MiSSP7 is able to repress the expression of several JA marker genes related to cell wall remodeling.

Because MiSSP7 is able to reduce JA-induced degradation of *PtJAZ6* and partly repress JA-induced gene transcription (Fig. 3), we wished to determine if MiSSP7 application could block the previously described inhibitory effect of JA on *L. bicolor* Hartig net development (19). The inhibition of Hartig net formation induced by exogenous MeJA is dose-dependent, with lower concentrations of MeJA (10^{-14} M) having no significant effect on the establishment of the Hartig net and concentrations higher than 10^{-12} M significantly inhibiting Hartig net formation (Fig. 4A). The inhibition of Hartig net establishment was not a result of reduced fungal growth rate caused by MeJA addition (Fig. S6).
the mutualistic ECM fungus *L. bicolor*, targets plant-encoded JAZ proteins and interacts with them in the nucleus of the plant. MiSSP7 is able to block the activity of MeJA and promote the proliferation of *L. bicolor* in plant tissues. This effect is likely a result of the ability of MiSSP7 to reduce the JA-induced degradation of the JAZ protein, thereby repressing JA-induced gene transcription. Finally, we demonstrate that the activity of MiSSP7 during the colonization process can be replaced by either inhibiting JA signaling *in planta* or through transgenic overexpression of the PtJAZ6 gene.

It is interesting that MiSSP7 acts to repress the expression of JA-induced genes in tissues as both biotrophic pathogens and mutualistic arbuscular mycorrhizal (AM) fungi promote JA accumulation in plant tissues to favor colonization (35). More recently, we made the observation that mutualistic ECM fungi are distinct from other biotrophic organisms in that JA inhibits their proliferation within root tissues (19). This finding is consistent with the earlier demonstration that colonization by another ECM fungus, *Paxillus involutus*, results in accumulation of the JA antagonist SA in root tissues and a decrease in JA biosynthesis...
the competitive exclusion of COI1 binding with PtJAZ6 (Fig. 3E), likely results in the observed repression of JA-induced gene regulation in poplar roots during colonization by L. bicolor. Of the JA marker genes repressed by MiSSP7, the majority have annotated functions relating to cell wall modification (e.g., extensin, pectin esterase, chitinase) (Fig. 3E and Table S1), suggesting that rather than inhibiting chemical defense induced by the JA pathway, MiSSP7 is affecting cell wall chemistry. The effect on cell wall modification suggested by these data is consistent with our earlier findings regarding the role of MiSSP7 during L. bicolor colonization of poplar roots (26). Within root rhizodermal cells this alteration to cell wall dynamics would then be sufficient to allow for the penetration of fungal hyphae into the root and establishment of the Hartig net. It is interesting to note, however, that some JA-inducible genes normally repressed during the L. bicolor colonization process of poplar roots (Fig. 3E) are not affected by MiSSP7 application. This finding would suggest that there are other effectors or mechanisms at play that target other aspects of the JA signaling cascade during the establishment of the ECM root tip. Taken together, these findings have a fundamental impact upon our understanding of how L. bicolor “negotiates” a symbiotic relationship by altering a plant’s ability to respond to JA. It remains to be seen if other mutualistic fungi similarly target JAZ-domain proteins and, thereby, affect the JA responsiveness of their host plant to foster symbiotic interactions.

Materials and Methods

Template cDNA for all cloning and YTH procedures was generated from RNA extracted using P. trichocarpa roots undergoing colonization by L. bicolor S238N after 0, 2, 4, 6, and 12 wk postcontact with the fungus. Yeast DNA reporter assay and YTH screens were performed as outlined previously (27). DiviVa and BiFC interaction tests were used to verify each interaction found in the YTH as per previous reports (28, 36).

An in vitro assay was used to determine the effects of different chemicals on the activity of L. bicolor S238N to colonize poplar roots after 2 wk of contact between the two organisms as per Felten et al. (33). To test if transgenically altering the transcription of PtJAZ6 could complement the loss of MiSSP7 in L. bicolor AT1007? RNAi mutants during mycorrhization, we used Agrobacterium rhizogenes strain 15834 to generate stably transformed roots of P. tremula × Populus alba 717–184 using a technique similar to that described in Chabaud et al. (37).

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24. Martin F, et al. (2012) Screening a cDNA library for protein-protein interactions directly targeting a symbiotic relationship by altering a plant’s ability to respond to JA. It remains to be seen if other mutualistic fungi similarly target JAZ-domain proteins and, thereby, affect the JA responsiveness of their host plant to foster symbiotic interactions.

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Supporting Information

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SI Materials and Methods

DNA Recombination Procedures. Template cDNA for all cloning procedures was created from RNA extracted using the RNaseasy Plant kit (Qiagen) according to the manufacturer’s instructions with the addition of 25 mg polyethylene glycol 8000/mL RLC buffer to the extraction solution from Populus trichocarpa roots undergoing colonization by Laccaria bicolor S238N. An on-column DNA digestion step with DNase I (Qiagen) was also included to avoid DNA contamination. RNA quality was verified by Experion HighSens capillary gels (Bio-Rad). Template cDNA for yeast II hybrid was prepared and amplified as described in ref. 1. Synthesis of cDNA for single gene cloning was synthesized from 100 ng of total RNA was performed using the iScript kit (Bio-Rad) according to the manufacturer’s instructions. For cloning procedures all primers were ordered from Eurogentec and PCR amplification was performed using Accuprime PfX Taq (Invitrogen) according to the manufacturer’s instructions and optimized according to each primer pairing. All primers were designed using L. bicolor genome v2.0 (http://genome.jgi-psf.org) and P. trichocarpa genome v3.0 (www.phytozome.net). All vectors, with the exception of the bioluminescence complementation (BiFC) vectors, used in this study were GATEWAY compatible (Invitrogen) and BP and LR cloning recombination reactions were performed according to instructions provided by the manufacturer (Invitrogen). Genes used in BiFC cloning were inserted directly into the vectors (pSATN-cCFP and pSATN-nVenus) using BglII and BamHI site using the In-Fusion recombination enzyme as per the manufacturer’s instructions (Clontech). Escherichia coli strain DH5α was used for all subcloning procedures.

Yeast One- and Yeast Two-Hybrid Analyses. Yeast one- and two-hybrid screens were carried out according to refs. 1 and 2. Yeast two mating were performed with MiSSP7 as bait protein against a cDNA library of P. trichocarpa roots colonized with L. bicolor at different stages of development. In each case, at least one-third of the mated cells (usually 1 x 10^6 to 3 x 10^6 zygotes) were plated on selective medium and analyzed for interacting proteins. For drop tests presented in Figs. 1, 2, and S1, yeast were pregrown overnight at 30°C with shaking. The following morning all cultures were adjusted to an OD600 of 1 after which they were serially diluted to 10^-2, 10^-3, and 10^-4 and plated on selective medium. Colonies were left to develop at 30°C for 3–5 d. To ascertain if coronatine would affect the level of the interaction between MiSSP7 (Mycorrhiza-induced Small Secreted Protein of 7 kDa) and PtJAZ6 or if MiSSP7 would interfere with the interaction between PtCOI1 and PtJAZ6, we grew Saccharomyces cerevisiae Mav203 colonies expressing MiSSP7 and PtJAZ6 on minimal selective medium (SC-L-W) supplemented with a range of coronatine concentrations (0, 15, 30, 50 μM) or S. cerevisiae Mav203 colonies expressing PtCOI1 and PtJAZ6 on medium supplemented with 15 μM coronatine and 0, 15, 30, or 50 μM MiSSP7 for 3 d in selective SD medium. MiSSP7 uptake into—and localization in—yeast cells was verified (Fig. S4C). After 3 d cells were supplemented with new media containing both chemicals to assure the presence of those chemicals. Yeasts were allowed to grow for another 24 h. Then OD600 was determined and cells were harvested (4,000 x g for 5 min), twice frozen in –80°C, and defrosted at 37°C. Thereafter, cells were resuspended in 150 μL Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KC1, 1 mM MgSO4, 50 mM β-mercaptoethanol) and 70 μL of 1% SDS were added. Hard vortexing for 30 s served to break the yeast cells. The assay was started by addition of 70 μL CPRG solution (10 mM in Z-buffer) and incubated at 37 °C. After reaction turned red, 60 μL of 1 M Na2CO3 was added to stop the galactosidase reaction. Time until color change was observed was recorded. After reaction was stopped, cells were centrifuged (4,000 x g, 15 min, 4 °C) and the absorbance of 200 μL supernatant at 570 nm was determined in a Tecan Infinity M200 Pro plate reader. The calculation of galactosidase units followed Clontech Yeast Handbook.

DivIVa Interaction Tests. In the DivIVA interaction test, one protein is constitutively expressed with a GFP-tag and the other protein is inducibly expressed with a DivIVA-tag (a tag that causes localization of the tagged protein to the poles of the E. coli cell). Before induction of the DivIVA-tagged protein, or in the case of a negative interaction between the two proteins, the GFP signal remains localized within the general cytoplasm of the E. coli. In the case of a positive interaction between the proteins of interest, however, the DivIVA-tagged protein together with the GFP-tagged protein (and thus the GFP signal) will relocate to the poles of the cell. Full-length genes of interest were cloned into either pNDIV, pCDIV, pNFGP, or pCGFP for the in vivo DivIVA interaction tests (3). For the interaction tests, E. coli strain BL21(DE3) (Invitrogen) were freshly cotransformed with two plasmids (one p*DIV and one p*GFP) and grown in Luria-Bertani (LB) medium supplemented with 15 μg/mL chloramphenicol and 50 μg/mL ampicillin for each test, and were grown at 37 °C for all tests. Positive colonies containing the two vectors were subcultured into fresh 1/2 LB medium supplemented with chloramphenicol and ampicillin and grown for 2–3 h. At this point, the cells were observed to ensure that the GFP-tagged protein was being expressed and was located in the cytoplasm, after which the DivIVA-tagged protein was induced with ρ-arabinose to a final concentration of 0.2% in LB medium. Cultures were induced for a maximum of 30–45 min and observed within this time-frame for relocation of the GFP marker. Images were captured with a Zeiss LSM710 scanning confocal microscope. A positive interaction was met if at least 50% of the cells exhibited GFP relocation to the poles of the bacterium.

In Vitro Mycorrhiza Formation. An in vitro assay was used to determine the effects of different chemicals on the ability of L. bicolor S238N to colonize poplar roots. In this assay, P. tremula × Populus alba clone 717–1B4 was rooted in between two cellophane membranes on the surface of solid MS medium (4). At the same time, new L. bicolor fungal colonies were grown on a cellophane membrane on Pachlewski solid medium [2.7 mM di-ammonium tartrate, 7.3 mM KH2PO4, 2.0 mM MgSO4·7 H2O, 13 mM maltose, 110 mM glucose, 2.9 μM thiamine-HCl, and 1 mL of a trace-element stock solution Kanieltra medium solidified with 1.2% (wt/vol) agar]. Once the poplar roots had achieved a length of between 2 and 3 cm, they were transferred onto a cellophane membrane on mycorrhization media low-glucose Pachlewski solid medium (control) or medium supplemented with a final concentration of 10^-8 M MeJA or jasmonic acid (JA), 100 μM aspirin or SHAM, and placed into direct contact with 10 d old L. bicolor or L. bicolor ΔmiSSP7 RNAi mutant colonies, as per Felten et al. (4). Concentrations of these compounds were chosen based on previous work which showed their biological activities at these dosages (5–8). To test if MiSSP7 could block the effect of MeJA, after 1 wk of direct contact between L. bicolor and P. tremula × P. alba
cloned into the 717 culture was used at an OD600 leaves were dosed with either Nicotiana benthamiana plants for leave infiltration studies were grown for 4–6 wk in a growth chamber with constant humidity (60%), 22 °C, and 16 h light. Agrobacterium tumefaciens strain GV3101 was transfected with a pMDC plasmid containing the full-length MiSSP7 sequence for PjAZ6 in phase with a GFP tag or a pTRBO vector containing cDNA of MiSSP7. The transformed Agrobacteria were grown overnight at 28 °C in 5 mL of YEB medium supplemented with Rifampicin (100 μg/mL), Gentamicine (10 μg/mL), and Kanamycine (50 μg/mL). After overnight growth, Agrobacterium cultures were pelleted by spinning at 4,000 rpm for 15 min and resuspended in an equal volume of infiltration buffer (10 mM MgCl2, 10 mM Mes, pH5.6, 200 μM Acetosyringone). Bacteria were left in the infiltration buffer for at least 2 h at 28 °C with gentle shaking in the dark. Each Agrobacterium culture was used at an OD600 = 0.1–0.2. After incubation, Agrobacterium suspensions for protein coexpression were combined and 4–6-wk-old N. benthamiana leaves were infiltrated. Between three and seven biological replications were performed. Agro-infiltrated plants were incubated 24–48 h at room temperature until GFP signal was visible in nuclei of epidermal cells. At this stage the leaves were then sprayed with 10−3 M MeJA (supplemented with 0.5% TWEEN 20) and left for an additional 24 h. Small fragments (0.5–1 cm2) of leaves were excised from the infiltrated area and mounted on slides with water or 60% (wt/vol) glycerol and analyzed with confocal microscope using the same exposure time and laser settings. For Western detection 5 cm2 of transformed leaf tissue was cut and frozen in liquid nitrogen. The presence of MiSSP7 within the transformed leaves was determined by RNA isolation and subsequent PCR-amplification.

**Stable Expression of PJAZ6 in Populus Roots.** To test if transgenically altering the transcription of PjAZ6 (either by over-expressing the gene under the control of a 3SS:: promoter or by reducing the level of transcripts using RNAi knockdown) could complement the loss of MiSSP7 in L. bicolor antispp RNAi mutants during mycorrhiza formation, we used Agrobacterium rhizogenes strain 15834 to generate transformed roots of P. tremula × P. alba 717–1B4 using a technique similar to that described in Chabaud et al. (10). The strain of A. rhizogenes was chosen based on its relatively high transformation rate (~50%) and the ability to produce roots with a normal architecture. Briefly, the coding sequence of PjAZ6 was cloned into the vector pH2GW7 for overexpression using the primer set: GGACAAGTTTTGTACAAAAAAGCAGGCTCGATGAGCCAATATGGCACAG (forward primer) and GGACCACTTTGTAACACATTTAAGCTCGAGC (reverse primer), and into the vector pH7GWIWG2(II) for RNAi knockdown using the primer set GGACCAAGTTTTGTACAAAAAAGCAGGCTCGATGAGCCAATATGGCACAG (forward primer) and GGACCACTTTGTAACACATTTAAGCTCGAGC (reverse primer). These vectors were transformed into A. rhizogenes strain 15834 and selected for on LB media supplemented with 75 μg/mL spectinomycin. To generate transgenic roots, 1-cm shoot cuttings of P. tremula × P. alba 717–1B4 were taken from 1.5-mo-old plants and a sterile syringe tip coated in transgenic A. rhizogenes was used to puncture the stem of the cutting several times. These cuttings were placed in MS agar media and left to root at 22 °C for 3 wk. In vitro mycorrhization experiments were carried out as above. Because the transformation process never yields 100% transformed roots, we also took samples of each root tested after the mycorrhization tests were completed and extracted the RNA (using the QIAgen RNAeasy Plant extraction Kit) and DNA (using the QIAgen DNAeasy Plant extraction kit) to ensure the integration of the T-DNA (Fig. S7 A and C) as well as the altered expression of PjAZ6 (Fig. S7 B and D), respectively. Within this paper, each transformed root is considered as an independent transformation event, and the development of the Hartig net between the three mycorrhizal root tips was used to calculate the SD within each independently transformed root.

**Transcriptional Analyses.** Two separate techniques were used for transcriptional analyses of MeJA and MiSSP7 effect on JA marker genes in poplar. For the effect of MeJA on the root transcriptome, roots were grown for 2 wk on MS medium supplemented with 10–8 M MeJA for 2 wk and then harvested, frozen in liquid nitrogen, and their RNA extracted using the RNeasy Plant kit available from Qiagen. RNA-Seq of two independent biological replicates for both MeJA treated as well as untreated, control roots, were sequenced using Illumina technology. Library construction and 100-bp paired-end reads sequencing was performed by IGA Technology services. Raw reads were trimmed for quality and aligned to P. trichocarpa cv4 v3 using CLC Genomics Workbench 6. For mapping, the minimum-length fraction was 0.9, the minimum similarity fraction 0.8, and the maximum number of hits for a read was set to 10. The unique and total mapped reads number for each transcript were determined, and then normalized to reads per kilobase of exon model per million mapped reads. A Baggerly test included in the CLC software was applied to the data. The samples are given different weights depending on their sizes (total counts). The weights are obtained by assuming a β-distribution on the proportions in a group, and estimating these, along with the proportion of a binomial distribution, by the method of moments. The result is a weighted t-type test statistic. The complete expression dataset is available as series (accession nos. GSE56865, GSE56863, GSE56864, and GSE53475) at the Gene Expression Omnibus at the National Center for Biotechnology Information (NCBI).

The analysis of the impact of colonization by L. bicolor and of MiSSP7 on these same marker genes was analyzed using data
obtained from microarray analyses. In all cases, RNA from three biological replicates was extracted as described above and cDNA was synthesized using the SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturer’s instructions for microarray analysis. Microarray experiments were performed as described previously (11). A Student t test with false-discovery rate (Benjamini–Hochberg) multiple testing correction was applied to the data using Cyber-T software (http://cybert.ics.uci.edu). Based on the statistical analysis, a gene was considered significantly induced if it met the following two criteria: (i) t test P value < 0.05; (ii) fold-change > 2. Before transcripts were declared present, the signal-to-noise threshold (signal background) was calculated based on the mean intensity of random probes present on the microarray. Cut-off values for signal intensity were then subtracted from the normalized intensity values. The highest signal intensity values observed on these arrays were ~65,000 arbitrary units. The complete expression dataset is available as series (accession nos. GSE56865, GSE56863, GSE56864, and GSE53475) at the Gene Expression Omnibus at NCBI (www.ncbi.nlm.nih.gov/geo).

Microarray results, the effect of Asp and SHAM treatment of the roots and the repression of JA marker genes by PtJAZ6 overexpression were verified using quantitative PCR. Briefly, cDNA was synthesized from 500 ng total RNA per sample using the iScript cDNA synthesis kit (Bio-Rad) following manufacturer’s instructions iScript kit. Real-time PCR was performed using a Chromo4 Light Cycler and OpticonMonitor Software. Real-time PCR analyses were performed using three biological replicates, with a technical replicate for each reaction using the SYBRGreen Supermix following the manufacturer’s instructions (Bio-Rad). Fold-changes in gene expression between treated and control roots were based on ΔΔCt calculations according to Pfaffl (12).

**Statistical Analysis.** At least three independent biological replicates were performed for each test outlined in this paper, unless otherwise noted, to ensure reproducibility and significance of data reported. A one-way ANOVA followed by a Tukey HSD (honest significant difference) multiple comparison test (P < 0.05) was used unless otherwise noted.

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Fig. S2. Phylogenetic tree of JASMONATE ZIM-DOMAIN (JAZ) proteins from 10 different plant species. The evolutionary history was inferred by using the maximum-likelihood method based on the JTT matrix-based model (1). The bootstrap consensus tree inferred from 500 replicates (2) is taken to represent the evolutionary history of the taxa analyzed (3). Branches corresponding to partitions reproduced in less than 30% bootstrap replicates are collapsed. Evolutionary analyzes were conducted in MEGA5 (3). Colored branches indicate groups of proteins homologous to a known Arabidopsis thaliana JAZ protein or the closely related TIFY domain-containing proteins PPD1 and PPD2. Red arrows indicate the placement of the two poplar JAZ proteins, PtJAZ5 and PtJAZ6, within this tree. All proteins are identified by either published name (for Arabidopsis thaliana) or by Phytozome annotation. POPTR, Populus trichocarpa; AT, Arabidopsis thaliana; Ccl, Citrus clementina; Cpa, Carica papaya; Csi, Citrus sinensis; Egr, Eucalyptus grandis; Glym, Glycine max; Mdo, Malus domestica; Medtr, Medicago truncatula; Ppe, Prunus persica.

Expression of different JAZ domain-containing genes are induced during *L. bicolor* colonization and by $1 \times 10^{-8}$ M JA treatment whereas the localization of MiSSP7 and PtJAZ6 is to plant nucleus. (A) Relative expression of the 13 JAZ domain containing genes in the *P. trichocarpa* genome in fine roots, in mature ectomycorrhizal (ECM) root tips and in $1 \times 10^{-8}$ M JA-treated ECM root tips as measured by whole-genome oligo-arrays. All tissues were grown under the same conditions and harvested after 2 wk postcontact between roots and *L. bicolor*. All values are the mean of three biological replicates. Gene annotations can be found in Table S1. The asterisks represent significant difference from control conditions ($P < 0.05$). (B) Root cells treated with 15 μM MiSSP7-FAM exhibit uptake of the fluorescent molecule and localization of the protein in the plant nucleus (examples indicated with arrows). (Scale bar, 10 μm.) (C) Transient transformation of *N. benthamiana* leaves with 35S::PtJAZ6-GFP construct via agroinfiltration results in production of GFP-tagged PtJAZ6, which localizes predominantly in the nucleus (examples indicated with arrows). (Scale bar, 40 μm.)
Fig. S4. Coronatine does not affect yeast growth nor does it alter the interaction between proteins unrelated to the JA pathway. (A) A comparison of maximal growth rate of yeast expressing MiSSP7+PtJAZ6 (black bars), PtJAZ6+PtCOI1 (white bars) or PtJAZ6+PtCOI2 (gray bars). (B) β-Galactosidase activity of yeast cells grown on differing concentrations of coronatine and expressing the control Krev1/RalGDS-wt interacting proteins of the ProQuest Yeast Two-Hybrid System (Life Technologies; ± SEM). (C) MiSSP7 is taken up into yeast cells and localizes to the nucleus. Fluorescently labeled MiSSP7 (MiSSP7-FAM; green signal) enters the cell and colocalizes with Hoechst 34580 (nucleic acid stain; blue signal) in the nucleus. (Scale bars, 5 μm.)
Fig. S5. *PtJAZ6* expression represses the transcription of JA-marker genes. (A) PCR proof of gDNA insertion of the 35S::*PtJAZ6* T-DNA construct into two independent transformant Populus lines (red arrow indicates product). (B) Quantitative PCR proof of overexpression of *PtJAZ6* in both transgenic lines. (C) Repression of five JA-marker genes compared with transcript levels in roots with normal levels of *PtJAZ6* expression. Black bars represent gene induction by MeJA, gray and white bars represent gene expression in lines 19 and 20 of 35S::*PtJAZ6*, respectively. Gene annotations can be found in Table S1 (± SEM).
Fig. S6. Treatment of *L. bicolor* with either JA, MeJA or JA signaling inhibitors (Asp and SHAM) does not affect fungal growth while variation of *PtJAZ6* expression alters *L. bicolor*’s ability to form a Hartig net. (A) Effect of different treatments on *L. bicolor* growth. (B) Transverse cross-section of control ECM roots with no altered expression of *PtJAZ6* exhibit normal mantle formation (M) and formation of a Hartig net (HN). (C) Transverse cross-section of *PtJAZ6-RNAi* ECM root with no visible Hartig net. (D) Transverse cross-section of 35S::*PtJAZ6* ECM root with normal Hartig net development. (Scale bars, 15 μm.) Parentheses show the depth of the Hartig net in B and D.
Fig. 57. Proof of gDNA insertion of PtJAZ6 T-DNA constructs and mis-regulation of PtJAZ6. (A) PCR proof of gDNA insertion of the 35S::PtJAZ6 T-DNA construct into 18 independent transformant Populus lines (denoted in main text as 35S::PtJAZ6 lines 1–18) and (B) subsequent quantitative PCR proof of overexpression of PtJAZ6. This graph is the expression of PtJAZ6 in colonized mutant root tissues compared with uncolonized control root tissues. (C) PCR proof of gDNA insertion of the PtJAZ6-RNAi T-DNA construct into nine independent transformant lines (denoted in main text as PtJAZ6-RNAi line 1–9) and (D) subsequent quantitative PCR analysis of the levels of PtJAZ6. This graph is the expression of PtJAZ6 in colonized mutant root tissues compared with uncolonized control root tissues. In B and D the dashed line represents the normal expression of PtJAZ6 in wild-type roots of poplar colonized by L. bicolor. Expression values over this line represent overexpression of the gene and expression values under the line represent reduced expression of the gene. +ve, PCR of the hygromycin resistance gene in the transformation vector.
Fig. S8. SHAM and Aspirin treatment of poplar root cells results in a repression of JA marker gene transcription. Expression of 5 JA-marker genes as induced by MeJA (black bars) over transcript abundance in untreated control roots. Expression of these same genes after cotreatment with MeJA + Aspirin (light gray bars) or with MeJA + SHAM (dark gray bars). Treatment with either Aspirin or SHAM significantly repressed the expression of these genes compared with MeJA treatment alone (*P < 0.05 ± SEM). Gene annotations can be found in Table S1.
Table S1. Correspondence between *P. trichocarpa* gene numbers and their homologs in *A. thaliana* used in this study

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